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(54) Title: MELANOCORTINS

(57) Abstract

The invention provides novel peptides derived from the group of hormones including ACTH. These so–called melanocortins can target different receptors which often have different localizations on several tissues. The presently invented peptides are useful for targeting receptors in the nervous system in an agonistic manner. Pharmaceutical compositions and uses are also disclosed.

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MELANOCORTINS

The present invention relates to the field of melanocortin peptides.

Melanocortins (which used to be called melanotropines also) are peptides originally derived from a larger precursor protein named pro-opiomelanocortin. The natural melanocortins share the heptapeptide core sequence Met-Glu-His-Phe-Arg-Trp-Gly. These melanocortins include α-MSH (α-melanocyte stimulating hormone), β-MSH, γ-MSH, γ-LPH (γ-lipotropin hormone) and ACTH (adrenocorticotrope hormone).

Melanocortins have a wide range of biological activities. They have been known for a long time to stimulate pigmentation and corticosteroidgenesis, but they have also been shown to induce excessive grooming behaviour in the rat, to stimulate conditioned active avoidance response, to increase blood pressure and heart rate, to accelerate nerve regeneration and to modulate immune responses. Quite recently five neuropeptide receptors for melanocortins have been identified and cloned. These receptors have different distribution patterns (in presence as well as in abundance) over the different tissue types. They belong to the family of so-called G-protein coupled receptors. Melanocortin receptor 1 (MCR-1) is expressed in melanocytes, whereas MCR-2 is the ACTH receptor expressed in for instance the adrenal gland. Melanocortin receptors 3, 4 and 5 have been found to be expressed in the central nervous system. The cognate ligands of these receptors have profound neuropharmalogical effects of, such as facilitated arousal, motivation, attention, memory and learning. The ligands have also been implicated in food-motivated behaviour. Further a relation with antipyretic activity has been disclosed.

Many different (synthetic) analogs of melanocortins have been prepared and suggested to be of use in activating or blocking one or more of the MC-receptors. This agonistic or antagonistic action has then been suggested to be useful in applications relating to pigmentation, nerve regeneration, etc.

specificity (selectivity) for the receptors expressed in the nervous system, and/or they lack in sufficient binding affinity or capability to induce the receptor mediated response or to block said response. Typically it would be desired that a drug targeting the MC-receptors be highly potent, orally administerable, reasonably resistant to breakdown in the body (have a sufficient half-life) and able to cross the blood-brain barrier.

The present invention provides peptides or peptide-like structures that meet the structural requirements to be useful as essentially MC-receptor specific drugs.

Thus the invention provides a peptide having specific binding affinity for a melanocortine receptor, preferably the mc3, mc4 or mc5 receptor comprising the sequence

X-Y-His-(D-2-Thienyl-Ala)-Arg-(Z)

or

X-Y-His-(3-pyridyl-D-Ala)-Arg-(Z)

whereby X and Y are amino acid residues and Z is an aromatic amino acid residue.

According to the present invention the above peptides are agonists for MSH activity which are highly potent. A very important contribution to the high potency can be attributed to the 7-position (counted as in the original ACTH-molecule) which should be D-2-thienyl-Ala or 3-pyridyl-D-Ala and for which only very limited and very similar residues may be substituted without losing the increase in agonist potency. Another important contribution to MSH agonistic activity is the omission of residues 1-3 and/or the omission of residues 11-13. A high contribution to activity is also provided by the presence of an aromatic amino acid residue at position 9. Positions 4 and 5 should normally not be omitted; these residues should be present though it is far less critical which amino acid residues are present at said positions. It is clear that at least conserved substitutions are allowed for these positions, but also less conserved substitutions will

histidine residue at position 6 and the arginine residue at position 8 are quite important for activity and should only be replaced by very conservative substitutions, if at all. Especially the more important residues in general should not be all replaced by substitutes in one and the same molecule. The preferred residue at position is Naftyl-alanine, be it in the D-or in the L-configuration.
The presence of this residue leads to a further increase in potency.
An amino acid residue at position 10 is not essential for the activity of the molecule, but it does seem to have some effect. If a residue is present it is preferred that this residue is glycine or lysine, whereby the latter has the additional advantage that it provides a reactive moiety which can be used to couple the peptide to other molecules or to make the peptide cyclic. In the event that a cyclic peptide is to be produced, which is preferred, since the half-life of such a cyclic peptide is improved over the half-life of the linear form, then a reactive moiety at the other end should also be provided. Another advantage of having a cyclic peptide is that these peptides tend to have a higher selectivity for MC-receptors, in particular an disulphide bridge increases the selectivity for the MC-4 receptor. Cyclic peptides can however also be produced by providing reactive moieties outside the essential core that enable closure of the loop, such as reactive moieties leading to a lactam.
The preferred residues at positions X and Y, (meaning 4 and 5 in the original ACTH-core) are Nle for X and Gly or Asp for Y, whereby the presence of Asp leads to a further advantage in having a reactive moiety for making a lactam.

The peptides according to the invention are generally more potent than MSH itself. The preferred peptides have potencies of up to 100 times the potency of MSH. Less potent peptides are within the scope of the present invention, since potency is not the only criterion which is required for a successful peptide-based drug. As already mentioned, half-life and selectivity are also important parameters.

the regular in vitro tests as well as an in vivo test in rats whereby the grooming behaviour is measured. The results in a grooming assay are good indications that the peptides will be able to activate the receptor and thereby the G-protein cascade coupled to said receptor and thus the peptides can be used as agonists for MC-receptors. Targeting the MC-receptor in particular of the MC-4 receptor for which a particularly selective peptide has been provided by the present invention in an agonistic manner is useful in the treatment of CNS-disorders, neurophathies, obesity, and in particular for diabetes related neuropathy, as well as neuropathy as a result of cytotoxic treatments (chemotherapy and the like).

Therefore the present invention also provides pharmaceutical compositions capable of treating the above conditions. Dosages for such treatments will usually be given once a day in doses of about 1 μg to about 100 mg per dosage unit, preferably 10 μg - 10 mg, more preferably 50 μg - 1 mg. The dosage should result in a concentration in the body of between 0,1 nm and 1 μm, preferably 1 nM - 100 nM, most preferably 10 - 50 nM. The compositions may comprise the usual additives for usual dosage formats for peptide drugs or for peptide derived drugs. The format is preferably an oral formulation such as a tablet, a granulate, a powder or a liquid formulation, although enteral and parenteral administrations may find application as well. Particularly preferred are compositions wherein a peptide according to the invention is combined with a drug aiming to prevent or that leads to neuropathy, such as insulin and cytotoxic agents.

The invention will be explained in more detail in the following experimental part.

Material and Methods

Melanocortin ligand receptor activity

Human embryonal kidney (HEK 293) cells were stably transfected with the human MC3(Gantz et al. JBC 1993. 268:8246-8250), human MC4 (Gantz et al. 1993. JBC 268:15174015179) or human MC5 receptor constructs using the calcium phosphate precipitation method. As a reporter plasmid 10 μg of the pCRE-LacZ vector (Chen et al. 1995. Anal.Biochem. 226:349-354), in which a cAMP responsive element drives expression of the LacZ gene, was transfected at the beginning of each experiment. The day after pCRE-LacZ transfection, cells were split in 96-wells plates. After 48 hours cells were stimulated with varying concentrations of the MC receptor ligands in assay medium (DMEM + 0.1 mg/ml BSA, 0.1 mM IBMX) for 6 hours. Cells were lysed in lysis buffer by a freeze-thaw round, substrate buffer (60mM sodium phosphate, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 200 μg/ml ONPG) was added and cells were incubated at 37°C for 1 hour. The activation of the cAMP signal transduction pathway upon receptor activation was detected in a micro plate reader (Biorad Model 3500) at 405nm using a colorimetric assay as described by Chen *et al.*)

Animals, implantation of cannulas, intracerebroventricular injection

Male Wistar rats weighing 120-130 g were implanted with cannulas into the foramen intraventriculare under hypnorm anaesthesia (Brakkee *et al.*, Life Science vol. 17 1979,). Rats were allowed to recover for 3 days and used for experiments during the next 10 days. In case that rats were used for more than one grooming experiment they were allowed to recover for at least 3 days between subsequent experiments. Peptides (15 ng) dissolved in 3 μg saline (154 mM NaCl) were injected i.c.v. by means of a Hamilton syringe. Grooming tests were performed according to (Gispen *et al*, Lab. Anim. Sci. 29 1975). Rats were placed into the observation boxes immediately after the injection. Observation started 15 min after the

15 sec over 50 min, thus the maximal grooming score for a rat is 200.

Synthesis of peptides

Purification of peptides

Preparative HPLC was carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cartridges plus quard cartridge (25x210 mm) filled with Delta-Pak C18 material. Peptides were detected at 230 nm using a Waters 486 spectrophotometer with a preparative cell. Purifications were performed in gradients using water with 0.1% trifluoroacetic acid (TFA) and acetonitrile with 0.1% TFA.

Methods for synthesis and cyclization of α-MSH peptides:

Multiple Peptide Synthesis

We used a Hamilton Microlab 2200 to synthesise up to 40 peptides simultaneously at 30 μmol scale. The Hamilton Microlab 2200 was programmed to deliver washing solvents and reagents to a rack with 40 individual 4 ml columns with filter, containing Rink (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy) resin for peptide synthesis. The columns were drained automatically after each step by vacuum. The coupling cycle was based on Fmoc/HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) chemistry [Fields et al., Peptide Research 4, 95-101] using double coupling steps of 40 minutes. Peptides were deprotected and cleaved in 2 hrs using 1.5 ml of a mixture of trifluoroacetic acid/phenol/thioanisole/water/ethanedithiol 10/0.75/0.5/0.5/0.25 and then precipitated twice by adding hexane/diethylether 1/1. The precipitate was dried and lyophilized from water/acetonitrile.

Cyclization of peptides

HPLC purified or crude peptides were used for cyclization via a disulfide bridge with cysteines or via a lactam bridge with the side chains of aspartic acid and lysine:

A. disulfide bridge: crude MBJ06 (40 mg) was dissolved in 40 ml of a 0.5% ammoniumbicarbonate solution at pH 8 and stirred overnight. After 24 hours no free sulphydryl groups were detected using Ellman's reagent and the product was lyophilized after addition of 0.5 ml of acetic acid. The peptide was dissolved in 3 ml of 40% acetic acid and purified by preparative HPLC in a gradient of 14% to 21% acetonitrile in water (containing 0.1% TFA) in 30 min. Yield after purification: 22.8 mg.

B. Lactam bridge: a mixture of 20 ml of DMF (peptide grade), 26 mg of Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, 0.05 mmol) and 0.017 ml of DIEA (0.1 mmol) was added to crude MBJ07 (20 mg, 0.012 mmol). The cyclization reaction was followed by analytical HPLC. After 2 hours the mixture was acidified to pH 4 using 0.1 M HCl. The product was purified by preparative HPLC after dilution with 30 ml of water in a gradient of 21% to 30% acetonitrile in water, containing 0.1% TFA, in 30 min. Yield 15.4 mg.

Results

Screening of modified MSH peptides generated by PEPSCAN® revealed several amino acids that increased MSH potency. D-2-thienyl-Ala and 3-pyridyl-D-Ala at position 7 of the MSH peptide was the most potent contributors of increased MSH potency. Naftyl-Ala at position 9 also increased MSH potency (figure 1b). Deletion of positions 1-3 and 11-13 further increased MSH activity. Figure 1 shows the dose-response curves for the following peptides:

MBU 23	*2GH6R7G#	linear
MBU 24	*2GH6R8G#	linear
MBU 27	*CGH6R8C#	cyclic disulphate
MBU 28	*2DH6R8K#	cyclic lactam
MBU 29	*2DH6R7K	cyclic lactam

* acetyl # carboxamid 2= Norleucine, 6= D-Thienyl, 7= 2-naftyl-L-alanine, 8= 2-naftyl-D-alanine

At position 9 an aromatic acid (F,W,Y) is highly preferred (figure 2). Figures 3 to 9 show the effect of the different amino acid substitutions at positions 4 to 10 of Nle-MSH, respectively.

The activity of these peptides on the induction of grooming behaviour following intracerebroventricular injections is shown in figure 6. 1500 ng is the lowest dose of MSH that induces excessive grooming behaviour. The five compounds tested here are 100x more potent than MSH in vivo.

Table 1. Calculated EC_{50} values for the three different cell lines

	hMC3	hMC4	hMC5
MBU 23	5.510^{-9}	5.510^{-9}	n.d.
MBU 24	3.010^{-9}	1.310^{-8}	4.810^{-8}
MBU 27	1.310^{-7}	6.010^{-9}	2.510^{-6}
MBU 28	6.310^{-7}	1.710^{-8}	4.510^{-8}
MBU 29	1.410^{-9}	1.510^{-8}	2.310^{-8}
α-MSH	1.410^{-8}	2.610^{-8}	3.010^{-8}

Legends

Figure 1. Dose response curves for the different MSH peptides on HEK 293 cells stably expressing human MC3, MC4 and MC5 receptors. MBU 27 shows specificity for the MC4 receptor. MBU 23 is a potent ligand on all three receptors.

Figure 1b. Effect of the different aminoacids at position 7 and 9 together with MBU 23 and MBU 24. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC receptors; MC3, MC4 and MC5 receptors. Values

activity of 100 nM NDP-MSH.

Figure 2. Effect of single aminoacid substitutions at aminoacid position number 9 of NDP-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (Trp) are depicted. Values represent the percentage activity as compared to the maximal activity of 10 nM α-MSH. All peptides were tested at 10 nM on HEK 293 cells stably expressing the three different MC receptors;MC3, MC4 and MC5 receptors. At this position on an NDP-MSH background an aromatic amino acid is highly preferred.

Figure 3. Effect of single aminoacid substitutions at aminoacid position number 4 of Nle-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (Met) are depicted. Values represent the percentage activity as compared to the maximal activity of 100 nM α-MSH. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC receptors; MC3, MC4 and MC5 receptors.

Figure 4. Effect of single aminoacid substitutions at aminoacid position number 5 of Nle-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (Glu) are depicted. Values represent the percentage activity as compared to the maximal activity of 100 nM α-MSH. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC receptors; MC3, MC4 and MC5 receptors.

Figure 5. Effect of single aminoacid substitutions at aminoacid position number 6 of Nle-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (His) are depicted. Values represent the percentage activity as compared to the maximal activity of 100 nM α-MSH. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC receptors;MC3, MC4 and MC5 receptors.

aminoacid position number 7 of Nle-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (Phe) are depicted. Values represent the percentage activity as compared to the maximal activity of 100 nM α-MSH. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC receptors; MC3, MC4 and MC5 receptors.

Figure 7. Effect of single aminoacid substitutions at aminoacid position number 8 of Nle-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (Arg) are depicted. Values represent the percentage activity as compared to the maximal activity of 100 nM α-MSH. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC receptors;MC3, MC4 and MC5 receptors. Only five aminoacid substitutions were tested.

Figure 8. Effect of single aminoacid substitutions at aminoacid position number 9 of Nle-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (Trp) are depicted. Values represent the percentage activity as compared to the maximal activity of 100 nM α-MSH. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC-receptors; MC3, MC4 and MC5 receptors.

Figure 9. Effect of single aminoacid substitutions at aminoacid position number 10 of Nle-MSH. On the X-axis the aminoacids replacing the endogenous aminoacid (Gly) are depicted. Values represent the percentage activity as compared to the maximal activity of 100 nM α-MSH. All peptides were tested at 100 nM on HEK 293 cells stably expressing the three different MC receptors; MC3, MC4 and MC5 receptors.

Figure 10.

Peptide induced grooming

3 µl saline, or 3 µl saline with either 15 ng MBU peptides or 150 ng α-MSH or 1500 ng α-MSH was injected i.c.v. in rats and grooming behaviour was scored (mean ± s.d.).

1. A peptide having specific binding affinity for a melanocortine receptor, preferably the mc3, mc4 or mc5 receptor comprising the sequence

X-Y-His-(D-2-Thienyl-Ala)-Arg-(Z)

or

X-Y-His-(3-pyridyl-D-Ala)-Arg-(Z)

whereby X and Y are amino acid residues and Z is an aromatic amino acid residue.

2. A peptide according to claim 1 comprising the sequence

X-Y-His-(D-2-Thienyl-Ala)-Arg-(2-naftyl-Ala)

or

X-Y-His-(3-pyridyl-D-Ala)-Arg-(2-naftyl-Ala)

wherein X and Y are as defined in claim 1.

3. A peptide according to claim 1 or 2 comprising the sequence

X-Y-His-(D-2-Thienyl-Ala)-Arg-(Z)-Z_2

or

X-Y-His-(3-pyridyl-D-Ala)-Arg-(Z)-Z_2

wherein X, Y and Z are as defined in claim 1 or 2 and wherein Z2 is an amino acid residue.

4. A peptide according to claim 3 wherein Z_2 is Gly or Lys.

5. A peptide according to any one of the aforegoing claims whereby Y is Gly or Asp.

6. A peptide according to anyone of the aforegoing claims wherein X is Nle.

7. A peptide according to any one of the aforegoing claims which is cyclic.

8. A peptide according to claim 7 whereby the cyclic peptide is produced by making an S-S bridge.

9. A peptide according to claim 7 whereby the cyclic peptide is produced by making a lactam.

10. A pharmaceutical composition comprising a peptide according to anyone of the aforegoing claims.

11. A composition according to claim 10, further comprising insulin or a functional equivalent thereof.

cytotoxic agent.

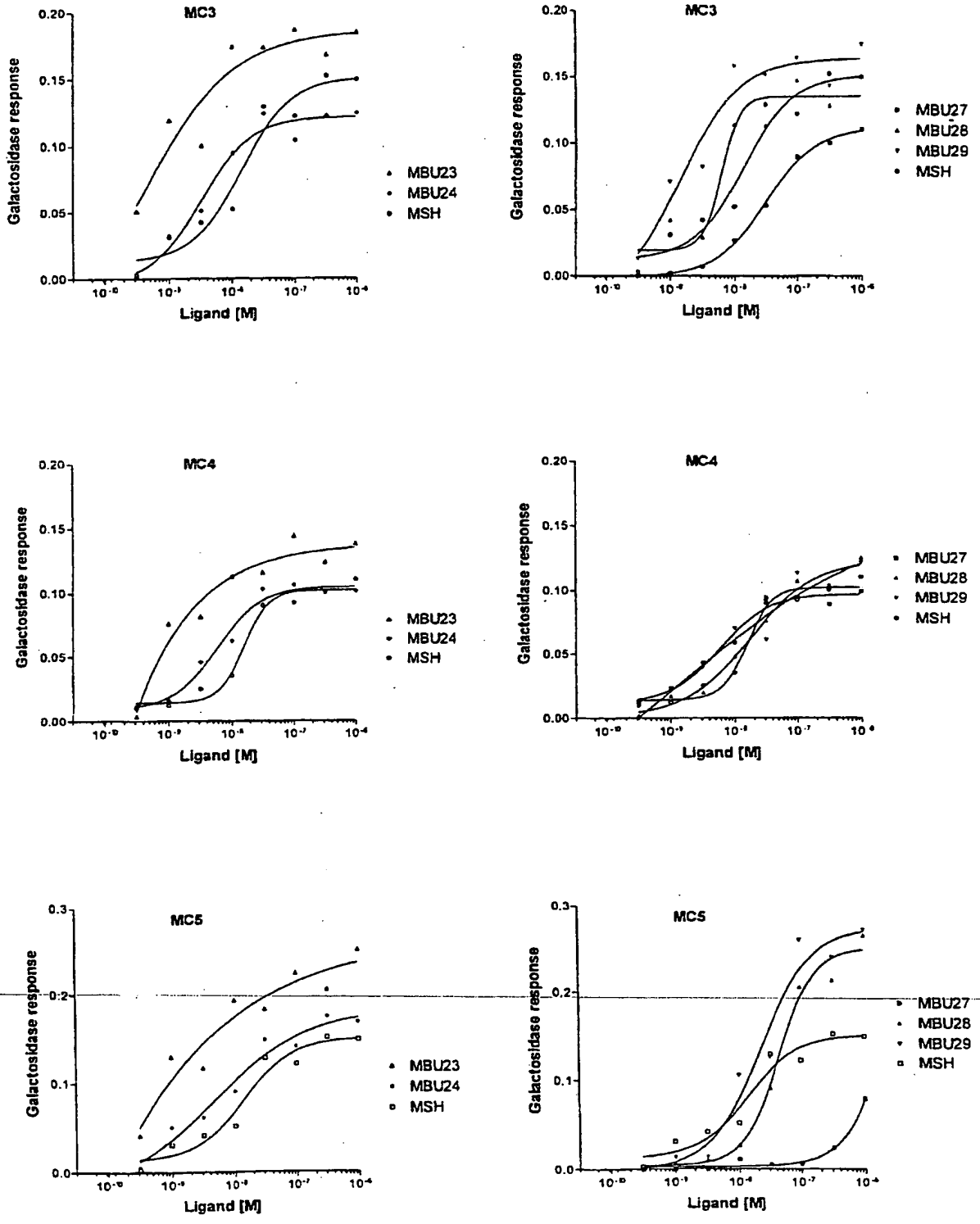
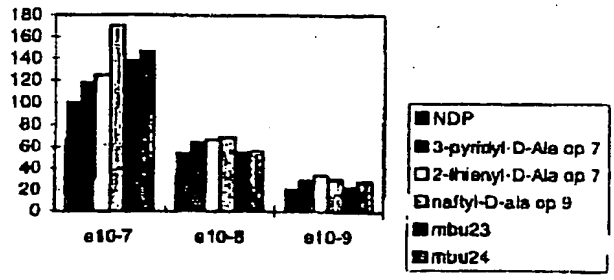
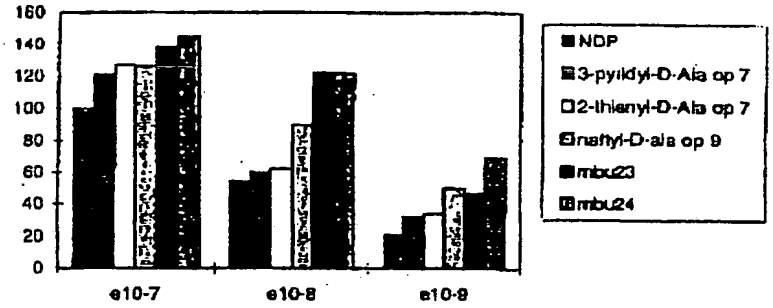

MC3
Galactosidase response
Ligand [M]
MBU23
MBU24
MSH
MC3
Galactosidase response
Ligand [M]
MBU27
MBU28
MBU29
MSH
MC4
Galactosidase response
Ligand [M]
MBU23
MBU24
MSH
MC4
Galactosidase response
Ligand [M]
MBU27
MBU28
MBU29
MSH
MC5
Galactosidase response
Ligand [M]
MBU23
MBU24
MSH
MC5
Galactosidase response
Ligand [M]
MBU27
MBU28
MBU29
MSH

FIG. 1

hMC3



hMC4



hMC5

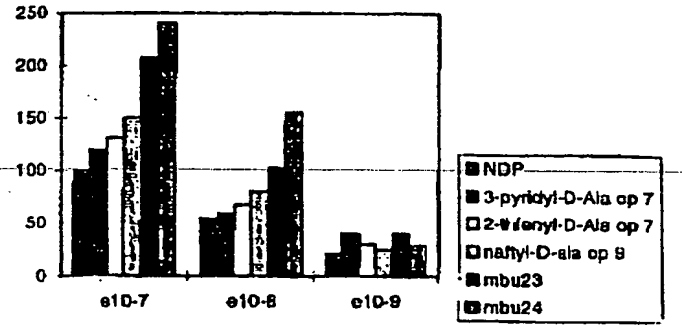
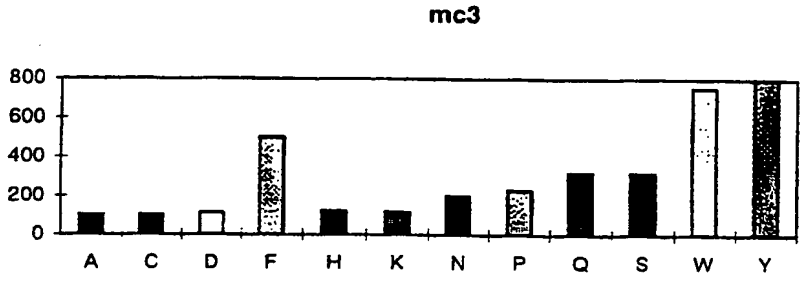
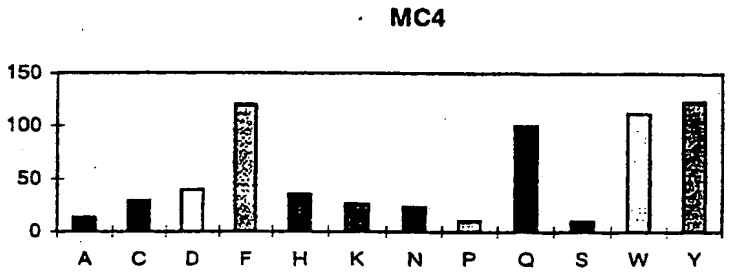


FIG. 1B


mc3
800
600
400
200
0
A C D F H K N P Q S W Y


MC4
150
100
50
0
A C D F H K N P Q S W Y

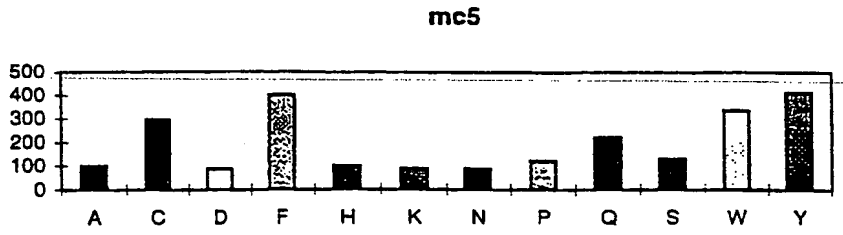

mc5
500
400
300
200
100
0
A C D F H K N P Q S W Y

FIG. 2

Position 4

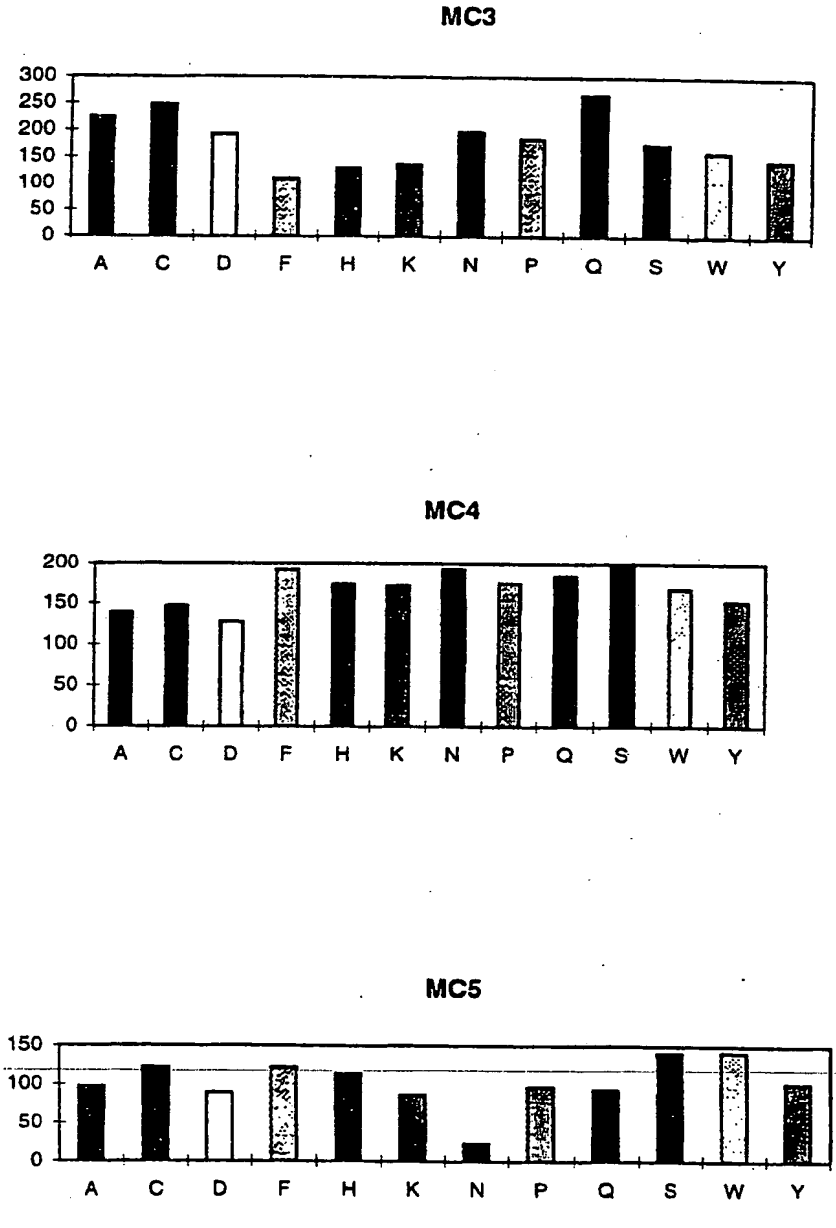
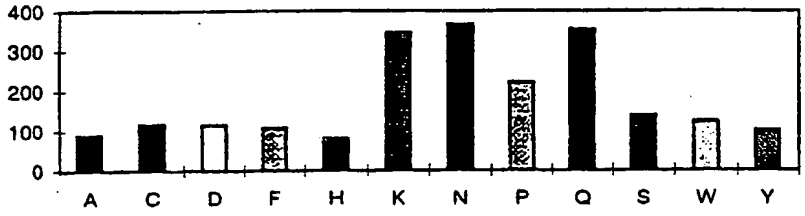


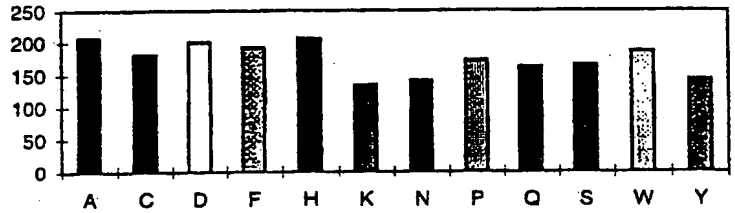
FIG. 3

Position 5

MC3



MC4



MC5

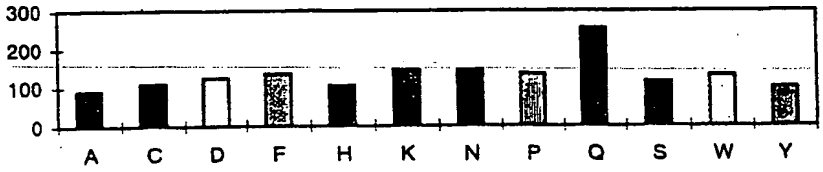
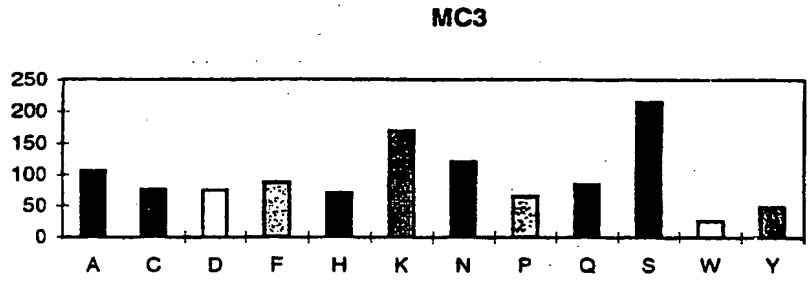
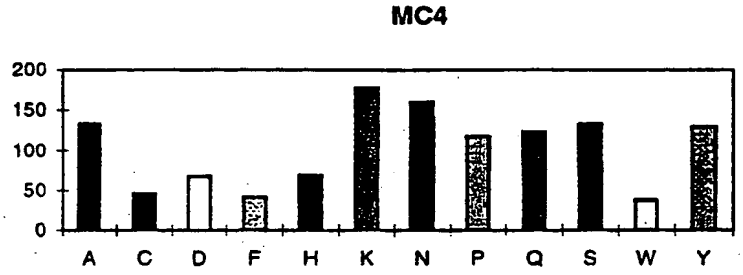


FIG. 4

Position 6





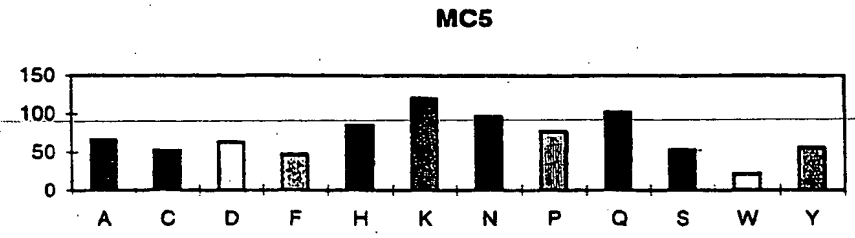
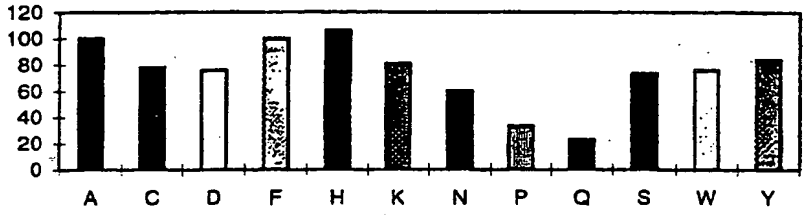


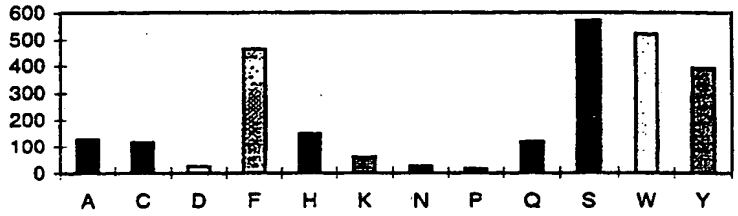
FIG. 5

Position 7

MC3



MC4



MC5

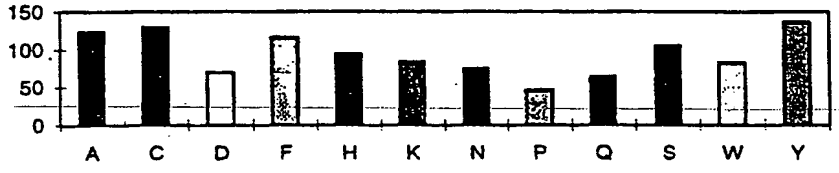
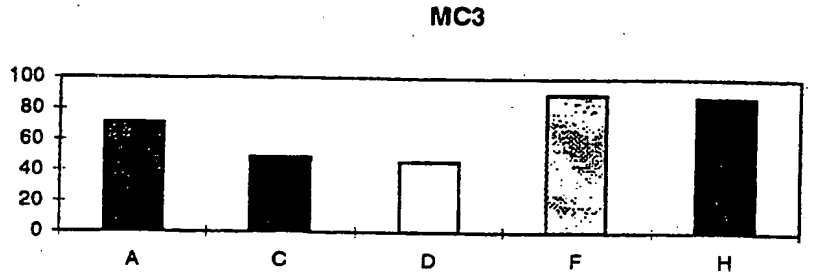
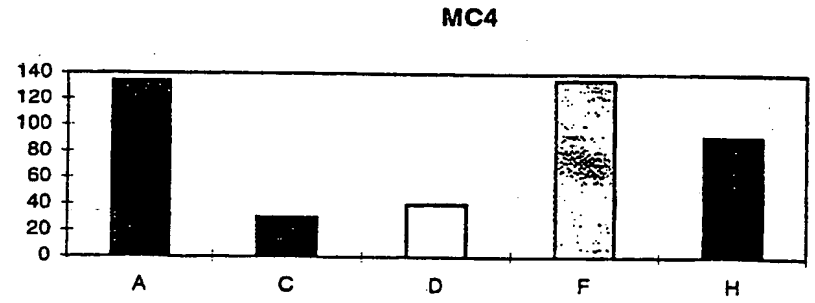


FIG. 6

Position 8





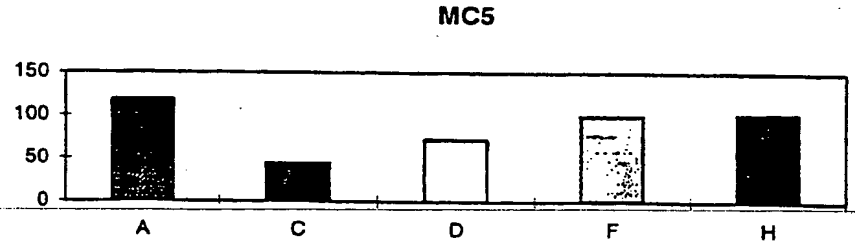
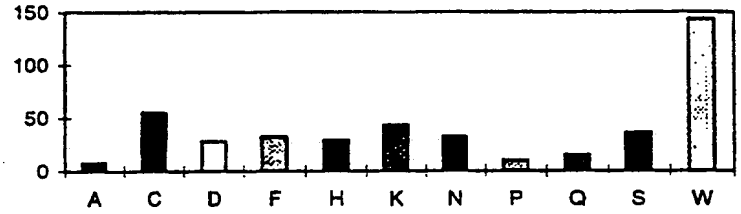


FIG. 7

Position 9

MC3

Position 9 MC4



MC5

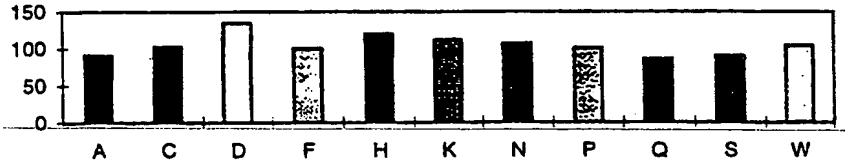
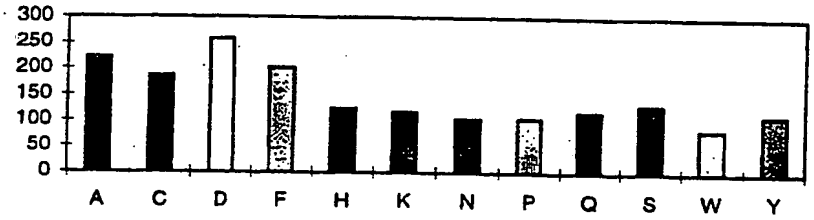


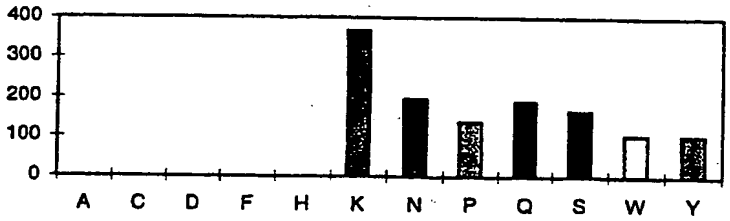
FIG. 8

Position 10

mc3



Position 10 MC4



mc5

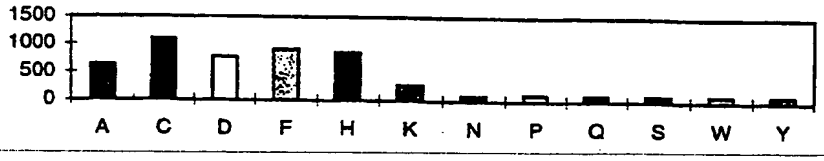


FIG. 9

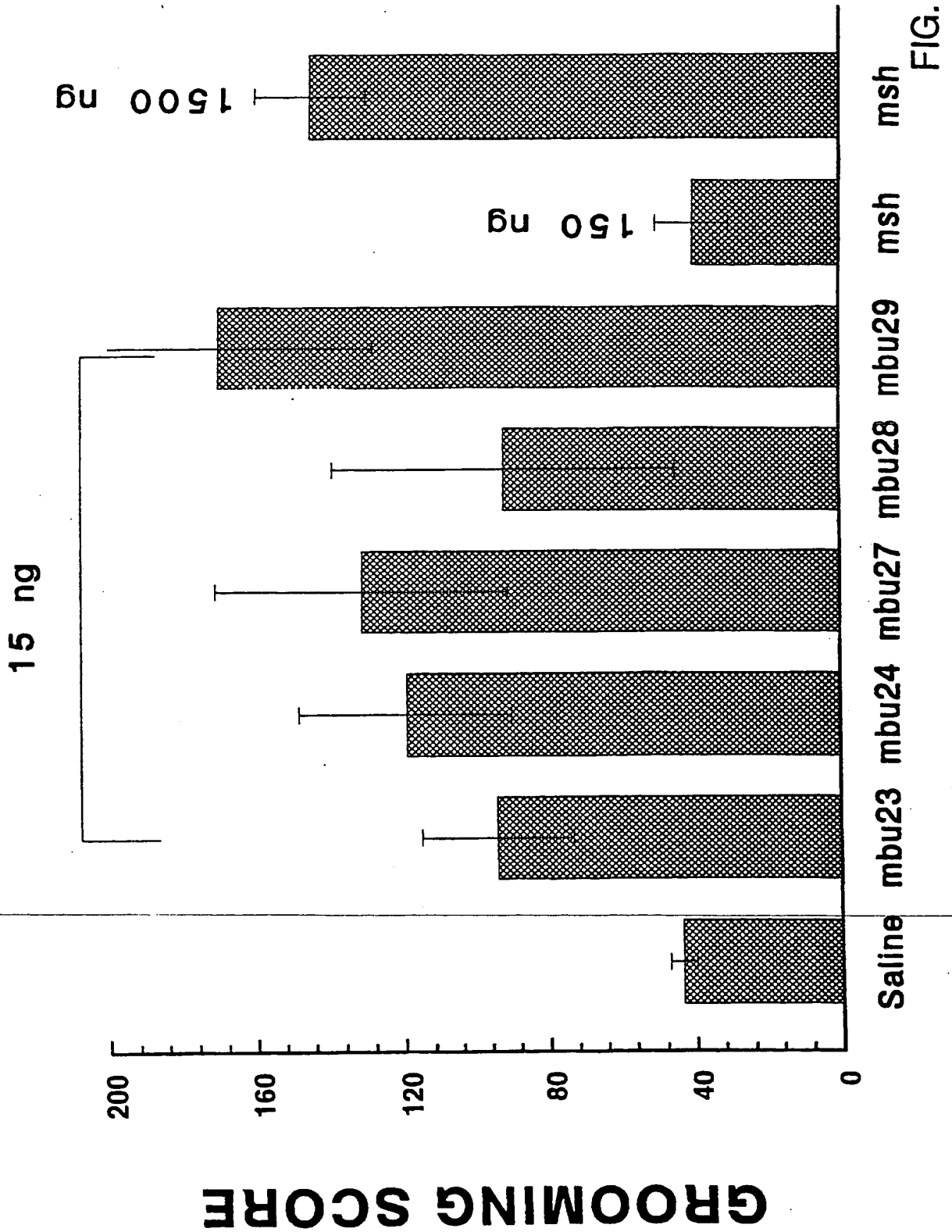

GROOMING SCORE
200
160
120
80
40
0
Saline
mbu23
mbu24
mbu27
mbu28
mbu29
msh
msh
15 ng
150 ng
1500 ng

FIG. 10

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(74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL–2587 BN The Hague (NL).

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(57) Abstract

The invention provides novel peptides derived from the group of hormones including ACTH. These so–called melanocortins can target different receptors which often have different localizations on several tissues. The presently invented peptides are useful for targeting receptors in the nervous system in an agonistic manner. Pharmaceutical compositions and uses are also disclosed.

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PCT/NL 97/00703

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A	R.A.H. HADAN ET AL.: "Identification of antagonists for melanocortin MC3, MC4 and MC5 receptors" EUROPEAN JOURNAL OF PHARMACOLOGY, vol. 269, no. 3, 15 April 1994, UTRECHT, NL, pages 331-337, XP002066333 see page 335, right-hand column, paragraph 2 - page 337, left-hand column, paragraph 1; table 1 -----	1

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/685 A61K38/34

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IPC 6 C07K A61K

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CODY, WAYNE L. ET AL: "Reversed-phase high-performance liquid chromatography studies of.alpha.-MSH fragments" J. CHROMATOGR. (1984), 314, 313-21 CODEN: JOCRAM;ISSN: 0021-9673, 1984, XP002066332 see compound XII see table I ---	1,3,4
A	WO 94 22460 A (UNIVERSITY PATENTS INC) 13 October 1994 see page 6, line 14 - line 30 see page 7, line 20 - line 29; claims; examples --- -/--	1,10

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